

REVIEW

Emerging applications of exosomes in cancer therapeutics and diagnostics

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Abstract

Exosomes are nanoscale extracellular vesicles that are shed from different cells in the body. Exosomes encapsulate several biomolecules including lipids, proteins, and nucleic acids, and can therefore play a key role in cellular communication. These vesicles can be isolated from different body fluids and their small sizes make them attractive in various biomedical applications. Here, we review state-of-the-art approaches in exosome isolation and purification, and describe their potential use in cancer vaccines, drug delivery, and diagnostics.

KEYWORDS

drug delivery, immunotherapies, nanobiology, patient-targeted therapies

1 | INTRODUCTION

Communication between cells is a critical process that occurs in all organisms. This sharing of information is facilitated either horizontal gene transfer by viruses¹ and bacteriophages² or by the secretion of soluble factors.³ Cells secrete extracellular vesicles (EVs), namely, apoptotic bodies, microvesicles, and exosomes,⁴ which can have significant impact on the local microenvironment as well as on distant tissues in the body.⁵ Apoptotic bodies are the largest among EVs, with sizes ranging from 50 to 5,000 nm, and contain DNA, RNA, and histone proteins. Apoptotic bodies are eventually removed by macrophages.^{6,7} Microvesicles (50–1,000 nm) are generally smaller than apoptotic bodies and are also known as shedding vesicles or exovesicles. Exosomes (30–120 nm; Figure 1),⁸ are the smallest among secreted vesicles, and consist of a lipid bilayer membrane that surrounds cytosol and other contents. Exosomes derived from human embryonic kidney cells have been observed to shrink in size when stored under 4°C temperatures.⁹ Exosomes typically do not contain cellular organelles but can carry different molecular constituents, including proteins and

nucleic acids, from the cell of origin.¹⁰ Proteins including those from the tetraspanin family (CD9, CD63, CD81, and CD82), ESCRT complex (TSG101, Alix)¹¹ and heat shock proteins (Hsp 60, Hsp70, Hsp90) are known to be found in exosomes; the composition of these proteins differs based on the cell or tissue of origin.^{12,13}

Vesicles secreted by cells can meet one of the following fates: (a) internalization by other cells in the immediate proximity, (b) internalization by cells at a significant distance away from the cell of origin, and (c) removal by distant tissues following entry into systemic circulation.¹⁴ Exosomes carry proteins and nucleic acids from their cell of origin,¹⁵ and these contents can be delivered to a different recipient cell leading to intercellular communication that can impact different physiological processes.^{5,16,17} Western blotting^{18–24} and fluorescence-activated cell sorting analysis^{22,25–27} of beads coated with exosomes have helped determine the presence of known cellular proteins in exosome preparations from different cells. In addition, mass spectrometry can be employed to identify unknown cellular proteins present in exosomes.^{27–30} Exosome-facilitated messages can regulate cellular growth, division, and apoptosis;^{31,32} changes in gene expression in

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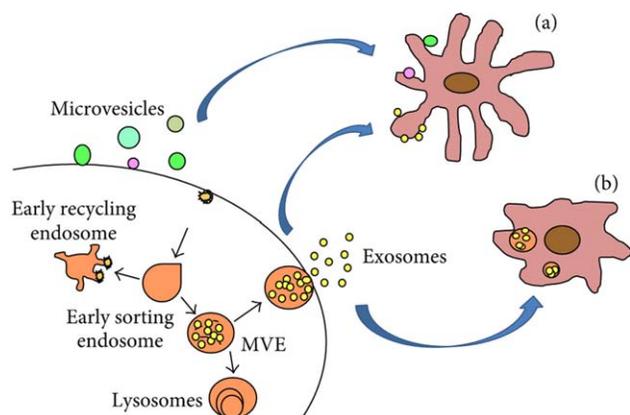


FIGURE 1 Schematic showing the release of exosomes and other vesicles from cells. Adapted from Ref. 8

recipient cells can be induced following delivery of multiple miRNAs by exosomes.³³ For example, exosomes derived from three different melanoma cell lines, while being morphologically very similar, had different effects on T-cell proliferation/growth suppression.³⁴ In this review, we first discuss cell sources that have been explored for obtaining exosomes and isolation methods that have been employed for obtaining enriched populations of these vesicles. We also discuss the use of exosomes as therapeutic carriers, disease biomarkers, and vaccines.

2 | CELL SOURCES FOR EXOSOMES

Cell lines including bEND3 and EL-4 are predominantly used for isolation of exosomes used in drug delivery due to the ease of their availability, the ability to scale up and the ability to generate exosomes of similar quality.^{35,36} Mesenchymal stem cells or hESC-MSCs are also excellent sources for isolation of exosomes,³⁷ and it has been observed that exosomes isolated from these are tolerated well by the immune system thereby making them an excellent choice for drug delivery.^{15,38,39} These cell lines can also be used to isolate exosomes for the purpose of developing anti-cancer vaccines. It is possible that primary cells isolated from patients/mice for developing these exosome-based anti-cancer vaccines might demonstrate a batch to batch variation in strength of the immune response.^{20,40} The use of exosomes for identifying biomarker levels mandates that patient-derived cells/body fluids are used. The presence/absence of disease can be determined by comparing exosomal biomarker levels of the patient with that of healthy controls.^{41–45}

3 | ISOLATION AND PURIFICATION OF EXOSOMES

Several methods have been investigated for the isolation and purification of exosomes from biological fluids.^{46,47} Centrifugation, filtration, immunological separation, microfluidic isolation,⁴⁸ and size-exclusion chromatography can be effectively applied in both laboratory research

and clinical medicine. Differential ultracentrifugation remains one of the most common techniques for exosome isolation^{49–51} and consists of several centrifugation steps that remove cells, large vesicles, and debris at lower centrifugation speeds. The pellets are discarded while the supernatant is subjected to higher centrifugation speeds in order to obtain exosomes as a pellet. Differential ultracentrifugation is commonly employed for the isolation of exosomes but the efficiency of the method is lower when plasma and serum are used due to higher viscosities of these fluids.^{24,47,52} Density gradient centrifugation combines differential ultracentrifugation with a sucrose density gradient. This method is mainly used for separating exosomes from nonvesicular particles including proteins and protein/RNA aggregates. The method allows separation of low-density exosomes from other contaminants but is highly sensitive to the centrifugation time.^{24,52,53}

Specific binding of antibodies to receptors present on the surface of exosome have been explored for isolating these vesicles from mixtures^{54,55}; in many cases isolated exosomes are subsequently analyzed for DNA or total RNA.^{56–60} As an application of this approach, antibodies are displayed onto magnetic beads to facilitate the specific binding and isolation of exosomes.^{61,62} The advantage of this immunoaffinity technique is the presence of various tetraspanins on exosomes isolated from different cell types which can be targeted using antibodies. The immunoaffinity technique employed was reported to be more effective at isolating exosomes compared to ultracentrifugation and density gradient methods.^{53,63} This method can be extremely effective for small-scale applications with low volume samples but the costs associated with scaling up this approach may be prohibitive for large scale isolation procedures.

Ultrafiltration has been investigated for the separation of exosomes from proteins,^{39,64,65} although the efficacy of this method has not been fully established for clinical samples. A porous membrane can be used for trapping exosomes resulting in their isolation from cell culture media. The filtration membrane helps concentrate the exosomal population. Recovery of exosomes from the membranes is facilitated by using ethanol or NaOH, which is typically followed by rinsing with phosphate-buffered saline.²⁴ However, application of large external force in this approach can result in deformation or damage of exosomes.

Polymer-facilitated precipitation, most commonly using polyethylene glycol, is also employed for recovering exosomes from mixtures (Figure 2).^{66,67} The main advantage of this method is the use of neutral pH. Although, there are no adverse effects on the isolated exosomes, contamination of exosomes with other nonexosomal materials is a significant drawback of this method.⁶⁸ In addition, the presence of the polymer may interfere with downstream analyses and/or usage.⁶⁹ Polymer-facilitated precipitation can be significantly improved by using methods for removing the polymer used in the operation. Size-exclusion chromatography is commonly used to separate macromolecules based on size,⁷⁰ and has also been investigated for exosome isolation (Figure 3).^{71,72} Exosomes isolated using this method are subjected to minimal shear force, resulting in relatively low damage to the structure of these vesicles; however, deformation of larger vesicles has been reported.⁷³

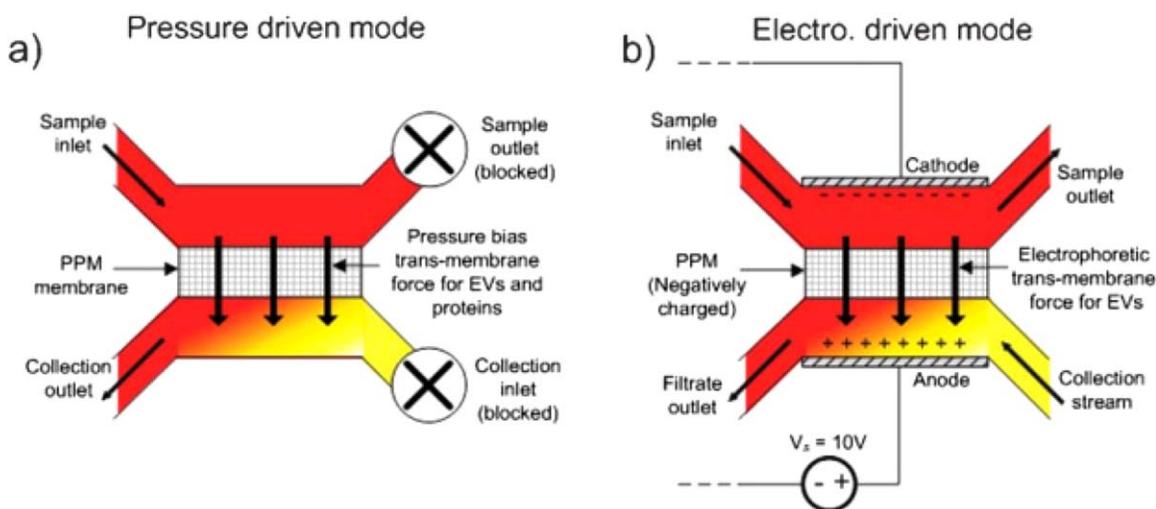


FIGURE 2 Schematic of the polymer-based precipitation method used for the isolation of exosomes. Adapted from Ref. 54

Exosomes isolated using ultracentrifugation—for example, using the ExoQuick approach—can result in increased yields compared to other methods, while also maintaining the quality of the exosomes isolated.²⁴ The swift isolation and higher exosome recovery enables analysis of protein expression in the recovered exosomes. Ultracentrifugation with density gradient centrifugation can improve the efficacy of the exosome purification without damaging the morphology. Variations in sizes of isolated exosomes have also been observed between fresh samples and those stored in DMSO and freezing also resulted in degradation of exosomal RNA over time.⁷⁴

4 | APPLICATIONS OF EXOSOMES

4.1 | Drug and nucleic acid delivery

The small sizes of exosomes make them attractive as vehicles in drug and nucleic acid delivery, although detailed molecular characterization of exosomes is necessary before adopting them in widespread applica-

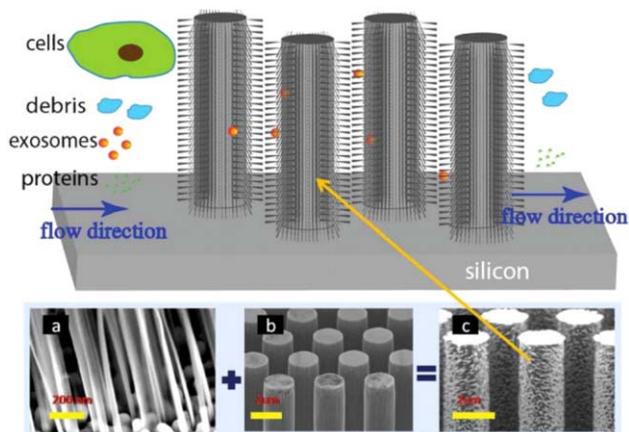


FIGURE 3 Schematic of the size-exclusion chromatography approach employed for the isolation and purification of exosomes. Adapted from Ref. 58

tions. Yang et al. hypothesized that exosomes derived from brain cells displayed brain-specific surface proteins which allowed them to pass through the blood-brain barrier (BBB) and deliver drugs across this barrier.³⁵ Exosomes were isolated from four different cell lines including brain endothelial cell line bEND.3, human glioblastoma cell line U-87 MG, human brain neuroectodermal cell line PFSK-1, and human brain glioblastoma A-172 cells. Rhodamine-123 (2 mg/ml), doxorubicin, or paclitaxel were independently loaded into different exosomes by mixing followed by incubation for 2 hr. Doxorubicin-loaded exosomes demonstrated higher efficacies for inducing death in U87-MG glioblastoma cells compared to paclitaxel-loaded exosomes. Exosomes isolated from bEND.3 cells were able to deliver the rhodamine-123 dye across the BBB following delivery via the cardinal vein of zebrafish embryos. Exosomes from bEND.3 cells were able to deliver drugs to a U-87 MG tumor grown in the brain of zebrafish and were also observed to inhibit VEGF (vascular endothelial growth factor) levels in vivo (Figure 4).³⁵ This, in turn, was able to induce a significant decrease in the tumor size, compared to treatments with the free drug. Intranasal delivery of curcumin or JSI-124 (cucurbitacin I) inhibitor-loaded exosomes was investigated as a potential therapeutic approach for brain inflammatory diseases.⁷⁵ Three different mouse models exhibiting lipopolysaccharide (LPS)-induced brain inflammation, autoimmune encephalitis, or the GL26 brain tumor model which exhibits inflammation due to infiltration of immune cells including macrophages and T-cells, were used in the study.⁷⁶ Exosomes were loaded by mixing curcumin with EL-4 (mouse lymphoma cell)-derived exosomes at a temperature of 22°C after which the loaded exosomes were separated using sucrose gradient centrifugation. Intranasally delivered exosomes were taken up by microglial cells, which are key mediators in neuro-inflammatory diseases; delivery of curcumin-loaded exosomes resulted in a reduction in activated microglial cells in both encephalitis and LPS-induced brain inflammation models. Intranasal delivery of exosomes loaded with the STAT3 inhibitor JSI-124 resulted in increased survival of mice with GL26 brain tumors. This study suggests that intranasal delivery of exosome-

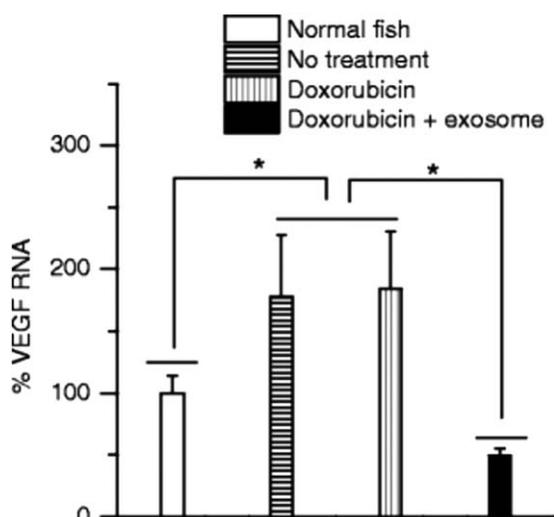


FIGURE 4 In vivo delivery of doxorubicin (Dox)-loaded exosomes across the blood brain barrier in zebrafish model. Significant inhibition of VEGF was observed in Dox-loaded exosomes compared to untreated controls and those treated with unencapsulated doxorubicin. Adapted from Ref. 35

encapsulated drugs could lead to a noninvasive approach for direct drug delivery to the CNS.⁷⁵

Exosomes isolated from RAW264.7 macrophages were loaded with catalase as a potential therapeutic approach for Parkinson's disease; catalase was loaded in order to potentially degrade reactive oxygen species in order to protect from inflammation.⁷⁷ Exosomes were loaded using four different methods—incubation with saponin, sonication, freeze-thaw, and extrusion. Sonication-loaded exosomes resulted in the highest uptake of catalase in PC12 neuronal cells in vitro as observed using fluorescence spectroscopy and confocal microscopy. Sonication was carried out using 20% power at 500V, 2kHz for 6 cycles pulsed for 4 s on and paused for 2 s. Extrusion was also employed for loading, and was performed by mixing catalase with exosome solution followed by 10 rounds of extrusion through a Avanti Lipids extruder.⁷⁸ Catalase-loaded exosomes, named exoCAT, protected neuronal cells from oxidative stress in vivo following intracranial injection into C57BL/6 mice. Biodistribution of exoCAT following intracranial injections indicated localization primarily in neuronal and microglial cells but also in astrocytes and endothelial cells.

Exosomes extracted from cow milk were employed for the delivery of therapeutic molecules against lung and breast cancer.⁷⁹ Exosomes loaded with withaferin-A were injected intraperitoneally into female athymic nude mice subcutaneously injected with A549 cells. A tumor inhibitory effect was observed with withaferin-A loaded exosomes at doses lower than those observed with the unencapsulated drug.

Exosomes isolated from dendritic cells from the bone marrow of C57BL/6 mice were employed for delivering small interfering RNA (siRNA) to the brain. A Rabies viral glycoprotein (RVG) peptide (single letter amino acid sequence: YTIWMPENPRPGTPCDIFTNSRGK-RASNG) was displayed onto the exosomal surface for targeting the acetylcholine receptor in the brain. Electroporation at 400 V and 125 μ F was used to load these exosomes with siRNA against GAPDH, and

the loaded vesicles were employed to investigate the knock down of GAPDH gene in C2C12 (murine muscle) and Neuro2A (neuronal cells). Gene knockdown efficacy using exosomes loaded with GAPDH siRNA was similar to that observed using lipofectamine. Exosomes were also well tolerated and did not induce strong immune responses in C57BL/6 and BALB/C mice. RVG exosomes were also able to deliver BACE1 siRNA across the BBB.⁸⁰

Exosomes derived from human bone marrow mesenchymal stem cells were investigated for delivering functional anti-miR-9 to glioblastoma multiforme cells⁸¹; communication between MSCs and brain glioblastoma cells can be mediated by exosomes.⁸² Flow cytometry and quantitative PCR (qPCR) indicated that MSC-derived exosomes, loaded with anti-miR9, led to the reduction of MDR1 expression in T98G and U87 glioblastoma cells. Delivery of anti-miR9 increased the sensitivity of these cells towards temozolomide, resulting in increased cancer cell death.⁸¹

Strategies for loading molecular cargo in exosomes and related efficacies differ based on the chemistry of the loaded molecule. The most common method for loading small-molecule drugs involves mixing the drug solution with a suspension of exosomes and incubating them at 25–37°C. The drug loading efficiency is typically determined using liquid chromatography.^{83,84} Loading of DNA onto exosomes using electroporation methods is likely restricted by the size of the exosomes with only large exosomes being capable of carrying large linear/plasmid DNA.⁸⁵ The loading efficiencies achieved using electroporation varied between 15 and 30% of those achieved with small molecule drugs and siRNA,^{86,87} Momen-Heravi et al. were able to achieve up to 55% loading efficiency using electroporation for miRNA molecules.⁸⁸ Smyth et al. achieved doxorubicin loading efficiencies of 5% by weight of exosomes using the mixing technique.⁸⁹ Similarly, Sun et al. observed a binding capacity of 2.9 g of curcumin for every gram of exosome using the mixing technique followed by sucrose density gradient centrifugation.³⁶ Kim et al. compared different techniques that is, mixing incubation, electroporation, and sonication for loading the drug paclitaxel into exosomes and observed 1.5, 5, and 29% loading efficiencies, respectively.⁹⁰ In comparison, Yang et al. were able to achieve 5% loading efficiency of siRNA in cationic lipopolymers with an encapsulation efficiency of 70%.⁹¹ and Cao et al. obtained 33% loading efficiency in calcium phosphate nanoparticles.⁹²

Taken together, these studies indicate the potential utility of exosomes in drug and nucleic acid delivery. The choice of cells from which exosomes are isolated, yield of exosome vesicles, cargo loading procedures, selection of targeting biomolecules (e.g., peptides) on the surface, biodistribution, and immune response are key factors for consideration for exosome-mediated delivery in future translational applications. Loading of small-molecule drugs can be efficient, although there is room for improvement in case of loading DNA/siRNA.

5 | BIOMARKERS

Exosomes play a vital role in cell-cell communication by directly engaging with surface ligands and/or by transferring their contents between

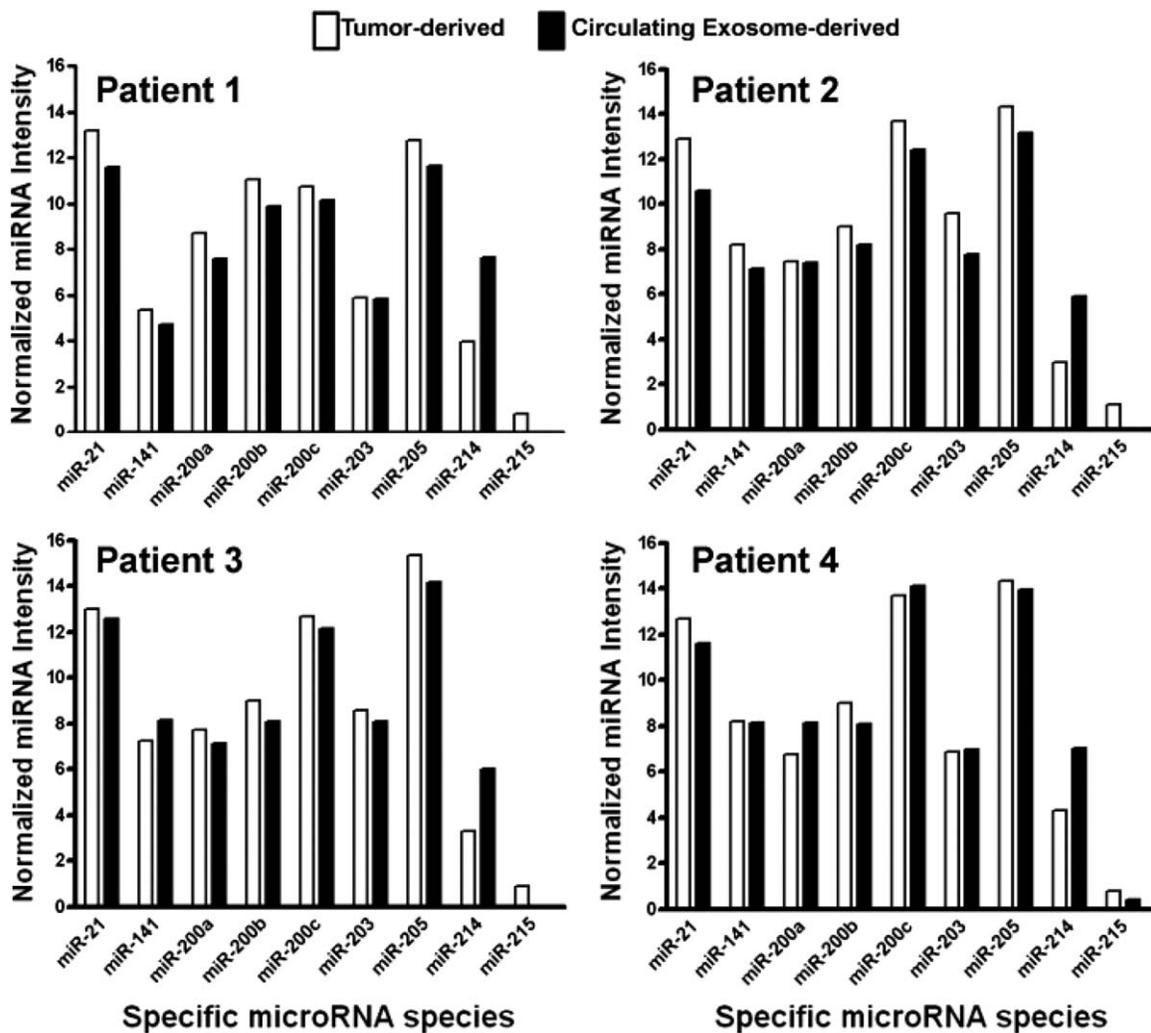


FIGURE 5 miRNA biomarker expression levels in tumor and exosome-derived samples showing comparable levels of miRNA between the two sample types. Adapted from Ref. 104

cells.⁹³⁻⁹⁶ Presence of exosomal RNA was implicated as evidence for horizontal transfer of genetic information between various cell types.⁹⁷⁻⁹⁹ Exosomes are thought to also transfer cellular mRNA as well as microRNA which indicates that tumor exosomes are functional and could suppress mRNA that codes for signal transduction components within T-cells.^{100,101} The RNA population in tumor-secreted exosomes includes microRNA and it is possible that this exosomal microRNA reflects the parental tumor signature. As a result, microRNA expression profiling can be useful as a diagnostic tool in diseases, including some cancers which lack definitive molecular biomarkers.

Exosome levels can be slightly elevated in benign tumors and highly elevated in cancerous patients as compared to normal controls; exosomes were isolated from the sera of patients/control subjects using magnetic activated cell sorting. Approximately, 175 different miRNA were found to be similar between tumor cells and exosomes. The up-regulated miRNA profile from exosomes also matched up-regulated miRNA profiles in ovarian cancer patients at different stages of the disease. However, this approach was not able to distinguish between different stages of cancer.¹⁰²⁻¹⁰⁵ Expression of miRNA in cir-

culating tumor-derived exosomes derived from lung adenocarcinoma patients were similar to that seen in primary tumors, indicating the potential use of these tumor-derived exosomes as biomarkers. In the future, it may be possible to analyze miRNA from circulating exosomes obviating the need for, or at least complementing, tumor biopsy samples¹⁰ in applications related to detecting disease, monitoring response to therapy, and investigating cancer recurrence.

The mRNA expression of two distinct biomarkers, PCA-3 and TMPRSS2, is found in exosomes and can be used to provide a direct link to the incidence of prostate cancer.¹⁰⁶ Proteins extracted from urinary exosomes can be of potential use in diagnostics of urinary tract diseases,⁴⁵ and prostate and bladder cancers. Eight urinary exosomal proteins were identified as biomarkers when patients with prostate, bladder cancer cells, and healthy cells were compared.¹⁰⁷ It has been reported in many cases that exosomal miRNA are prospective biomarkers for renal fibrosis and cardiovascular diseases.¹⁰⁸⁻¹¹⁰

Potential advantages of using exosomes as biomarkers includes the ability to reduce the use of invasive surgery for monitoring disease. Exosomes can be isolated from serum samples and have been

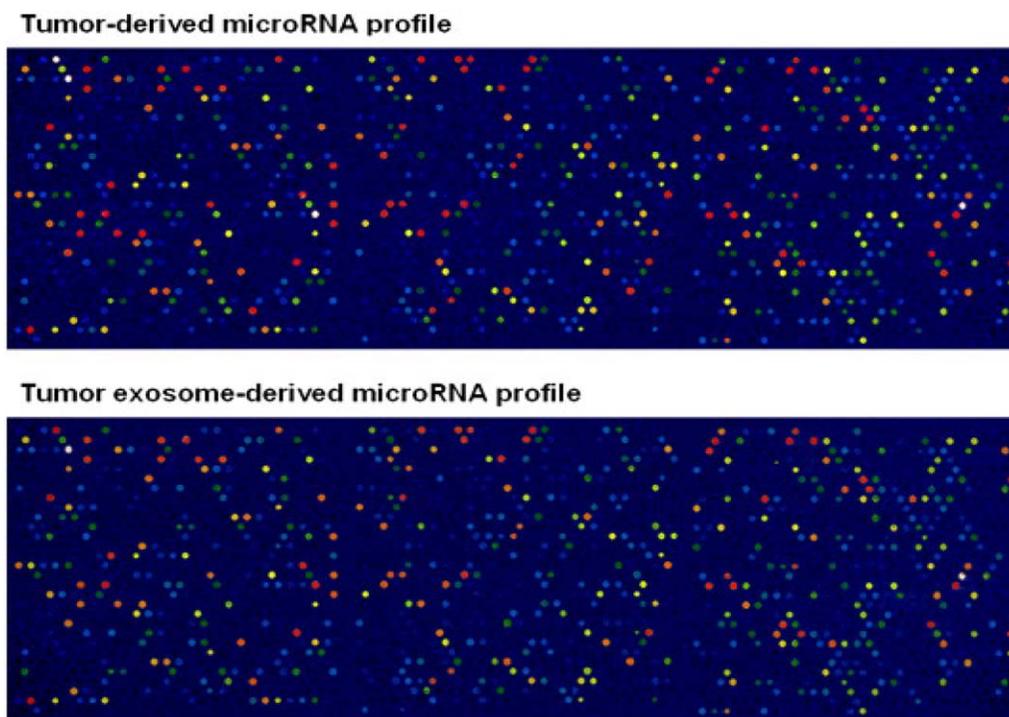


FIGURE 6 miRNA profile comparison between tumor and circulating exosomes. Adapted from Ref. 104

successfully used for biomarker detection in ovarian, lung and pancreatic cancers.¹¹¹⁻¹¹³ Exosomal miRNA has also been found to be useful in distinguishing between pancreatic carcinomas from benign pancreatic tumors and chronic pancreatitis¹¹³ and in detection of miRNA (miR-34A) responsible for conferring drug resistance to prostate cancer.⁴¹ Exosomal miRNA appear to be stable and can undergo multiple freeze-thaw cycles, variations in pH and heating without undergoing degradation or loss of expression levels; the miRNA profile is also highly specific to cancer/tumor tissue.¹¹³ However, a key drawback of using exosomes as biomarkers markers is that they cannot determine the severity of the disease because the miRNA levels can be identical during different stages of the disease as shown by Taylor et al. for ovarian cancer and Rabinowitz et al. in the case of lung cancer.^{104,112} The potential of using exosomal markers for clinical diagnostics needs to be further investigated in depth because various exosomal components including lipids, proteins and miRNAs can be promising in disease diagnostics.

6 | ANTICANCER VACCINES

Exosomes derived from specific sites in the body can be promising candidates for anti-cancer vaccines since they can present antigens against that specific type of cancer. Exosomes can be isolated from three sites of origin ascites (ascite-derived exosomes or AEX), dendritic cells (dendritic cell-derived exosomes or DEX), and tumors (tumor-derived exosomes or TEX).¹¹⁴⁻¹¹⁷ The antigens in these exosomes can elicit an immune response via MHC class I or class II molecules on CD8⁺ or CD4⁺ T cells. These exosomal MHC class I/II molecules on exosomes are likely used for communication with specific cell types.^{118,119} Mela-

noma antigens in DEXs were used to prime cytotoxic T lymphocytes in order to elicit an immune response through the Mart1 specific pathway in patients with high-grade melanoma.^{21,120} In a different study, exosomes were isolated from four different pancreatic cancer cell lines, SOJ-6, BxPC-3, MiaPaCa-2, and Panc-1. Exosomes derived from SOJ-6 and BxPC-3 cells were more effective at reducing growth human pancreatic adenocarcinoma cells compared to those derived from MiaPaCa-2 and Panc-1 cells; regulation of Hes-1 protein via downregulation of the Notch-1 signaling pathway and activation of mitochondria-dependent apoptosis pathway played a key role in the cell ablation efficacy.¹²¹

Dendritic cell-derived exosomes were shown to elicit Natural Killer (NK) cell responses following intradermal injection in C57BL/6 mice. Exosomes isolated from normal volunteers were primed with melanoma associated antigens MAGE3.A1 and MAGE3.DP04, and were used as vaccines in stage IIIb and stage IV melanoma patients. The treatment resulted in a regression of the cancer; while no changes in cytotoxic T-lymphocyte levels were observed, the level of NK cell increased with treatment. However, the NK cells obtained from patients that responded to the DEX treatment were more effective at killing K562 cells in culture compared to those obtained from nonresponders.^{115,122}

These studies indicate potential applications of exosomes in cancer immunotherapy. However, exosomes generally need mature DCs to elicit a T-cell response, since antigens present on exosomes need to be taken up by the dendritic cells before they can activate T-lymphocytes. This can be avoided by the use of adjuvants in some cases, which can allow exosomes to directly prime T-lymphocytes. In addition, the reliance on an intact immune system can restrict their use in

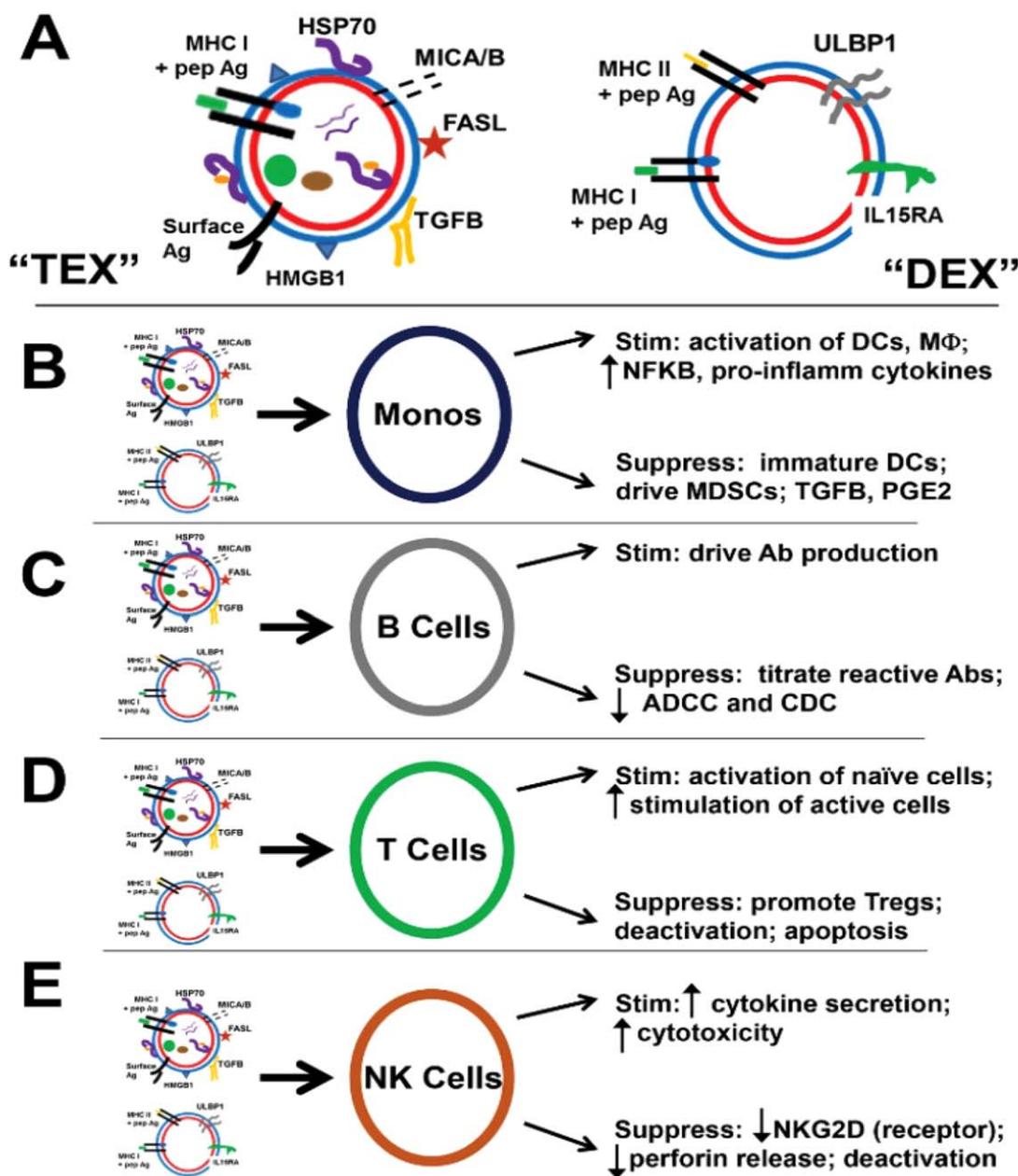


FIGURE 7 Interactions of dendritic cell-derived exosomes and tumor-derived exosomes with inflammatory cells. Reproduced with permission from Creative Commons. Adapted from Ref. 117

immunocompromised or immunosuppressed patients. However, this approach can elicit an immune response in already developed cancer which can induce regression of the tumor.¹²² This approach may also find less resistance from the suppressive nature of the tumor microenvironment,^{116,117,122–126} which can be a significant advantage.

7 | FUTURE DIRECTIONS

The potential for exosomes in the field of drug delivery is significant owing to their ability to selectively express proteins like tetraspanins which may allow for cell targeting. It will be necessary to obtain highly pure formulations of exosomes with low amounts of protein aggre-

gates and other microvascular particles. Optimization of ultracentrifugation coupled with density gradient centrifugation may offer one route toward obtaining enriched populations of exosomes. Further advancements that enhance the loading of drug/nucleic acid are necessary for effective delivery of these therapeutic molecules. Improvements in analytical methods and advances in biomarker discovery can facilitate the use of exosomes in disease detection.

8 | CONCLUSIONS

Exosomes are starting to gather attention in cancer therapeutics and diagnostics, with several applications in drug delivery, tumor

immunotherapy, and diagnostic biomarkers. Their unique strengths include enhanced passive targeting due to small size, indigenous nature, and the ability to cross biological barriers. However, the cumbersome nature of the methods required for isolation/purification, inability to distinguish between different cancer stages, and incomplete understanding of their impact on the immune system are some of the current limitations with this technology. It is anticipated that sophisticated engineering and detailed clinical studies that address these limitations will lead to the translation of exosome-based technologies in the future.

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